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Differential Activation of the *tcpPH* Promoter by AphB Determines Biotype Specificity of Virulence Gene Expression in *Vibrio cholerae*

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Vibrio cholerae **strains of the classical biotype express the genes encoding cholera toxin (CT) and toxin-coregulated pilus (TCP) under a variety of environmental conditions in vitro, whereas El Tor biotype strains express these genes only under specialized culture conditions. We show here that a single base-pair difference at positions −65 and −66 of the classical and El Tor *tcpPH* promoters, respectively, is responsible for the differential regulation of virulence gene expression in these two disease-causing biotypes. Analysis of *tcpP-lacZ* fusions in both *V. cholerae* and *Escherichia coli* indicated that transcriptional activation of the El Tor *tcpPH* promoter by the LysR regulator AphB was significantly reduced relative to that of the classical promoter. Reciprocal exchange of the *tcpPH* promoter between the two biotypes in *V. cholerae* showed that the ability to activate the transcription of *tcpPH* is not dependent on the biotype of the strain per se but on the *tcpPH* promoter itself. Classical and El Tor *tcpP-lacZ* promoter chimeras in *E. coli* localized the region responsible for the differential activation of *tcpPH* by AphB to within 75 bp of the transcriptional start site. Individual base-pair changes within this region showed that the presence of either an A or a G at position −65 or −66 conferred the classical or El Tor, respectively, pattern of *tcpPH* activation by AphB. Reciprocal exchange of these base pairs between biotypes in *V. cholerae* switched the biotype-specific pattern of expression of *tcpPH* as well as the production of CT and TCP in response to environmental stimuli.**

Two serotypes of *Vibrio cholerae*, O1 and O139, are capable of causing the life-threatening epidemic disease cholera. These strains have acquired a large pathogenicity island, TCP-ACF (20) or VPI, for *Vibrio* pathogenicity island (18), which has recently been reported to be the genome of a filamentous phage, VPIϕ (19). Carried on this element are a number of genes required for the expression and production of the primary colonization factor of *V. cholerae*, the toxin-coregulated pilus (TCP) (35). Disease-causing strains of *V. cholerae* have also acquired the genome of a second filamentous phage, CTXϕ (37). Within this element are the genes encoding the subunits of cholera toxin (CT), which is responsible for the profuse diarrhea associated with the disease. Strains capable of elaborating TCP apparently acquired CTXϕ by virtue of the fact that TCP serves as the receptor for the phage (37).

The expression of the genes encoding TCP and CT is positively activated by the AraC regulator ToxT (4, 9, 15). ToxT is itself encoded within the TCP-ACF pathogenicity element, and its expression is dependent upon two transcriptional activator pairs, ToxRS and TcpPH, as well as by stimuli from the environment (7, 13). ToxR and TcpP are homologous transmembrane DNA binding proteins which cooperate to activate *toxT* transcription (13, 26). The abilities of ToxR and TcpP to activate transcription are enhanced by the accessory transmembrane proteins ToxS and TcpH, respectively (7, 13, 24). The *toxR* and *toxS* genes, which are expressed as an operon, are not carried on either the TCP-ACF or the CTX element and have other important regulatory roles in *V. cholerae* (6, 25). The *tcpPH* operon is located within the TCP-ACF element, immediately upstream of the gene encoding the major pilin subunit, *tcpA* (28). TcpP and TcpH have no additional known roles in *V. cholerae* other than their involvement in *toxT* transcription. The *toxT* gene is located within the *tcpA* operon, and its expression is influenced by ToxRS and TcpPH at a promoter located immediately upstream of the gene (14) as well as by a promoter upstream of *tcpA* which appears to function in an autoregulatory capacity (1, 38).

The expression of the *tcpPH* operon is itself under the control of two regulatory proteins, AphA and AphB, which function synergistically to activate transcription (21, 33). AphB is a member of the LysR family of transcriptional regulators, and AphA presently has no known homologs. Neither *aphA* nor *aphB* is carried on the TCP-ACF or CTX elements and, like *toxR* and *toxS*, these genes presumably have other regulatory roles in *V. cholerae*.

The expression of *tcp*, *ctx*, *toxT*, and *tcpPH* is influenced by environmental stimuli, such as pH, temperature, and osmolarity (3, 9, 25, 35). The mechanisms involved in this environmental regulation are different between the two disease-causing biotypes of *V. cholerae* O1, classical and El Tor. Classical biotype strains typically exhibit maximal expression of these virulence genes in vitro in Luria-Bertani (LB) medium (pH 6.5) at 30°C, whereas El Tor biotype strains require a bicarbonate-containing medium (AKI medium) at 37°C for high-level expression (16). This differential regulation of virulence genes between the two biotypes may account for why infections with classical strains are generally more severe than those with El Tor biotype strains (17).

The overproduction of AphB in El Tor biotype strains has been shown to increase the expression of *tcpPH* and *tcpA* to close to classical levels in LB medium (pH 6.5) at 30°C (21). Since the expression of *aphB* is similar in both biotypes (21), these results suggested that some aspect of AphB function was involved in the differential regulation of virulence genes between the biotypes. We show here that the molecular basis for the biotype specificity of virulence gene regulation is a single base-pair difference 65 (classical) or 66 (El Tor) bp upstream from the *tcpPH* transcriptional start site, which is critical for activation by AphB.

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TABLE 1. Bacterial strains and expression plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strains		
V. cholerae		
KSK618	CG842 *tcpP-lacZ* (classical Ogawa Sm^r)	33
KSK805	KSK618 Δ*aphA1* Δ*aphB1*	21
KSK725	KSK262 *tcpP-lacZ* (El Tor Inaba Sm^r)	21
GK184	KSK725 Δ*aphA1* Δ*aphB1*	This work
GK250	KSK725 Δ*tcpPH* promoter	This work
GK300	KSK618 Δ*tcpPH* promoter	This work
GK318	GK300 with classical *tcpPH* promoter (C/C control)	This work
GK319	GK300 with El Tor *tcpPH* promoter (C/E hybrid)	This work
GK321	GK250 with classical *tcpPH* promoter (E/C hybrid)	This work
GK323	GK250 with El Tor *tcpPH* promoter (E/E control)	This work
GK404	GK300 with classical *tcpPH* promoter A→G change at position −65	This work
GK436	GK250 with El Tor *tcpPH* promoter G→A change at position −66	This work
KSK218	CG842 *ctx-lacZ* Sm^r Cm^r	32
KSK1019	KSK218 with TAA codon in *tcpP* coding region	This work
O395	Classical Ogawa Sm^r	Laboratory collection
KSK1093	O395 *tcpPH* promoter A→G change at position −65	This work
C6706 str2	El Tor Inaba Sm^r	Laboratory collection
KSK1117	C6706 str2 *tcpPH* promoter G→A change at position −66	This work
E. coli		
MC1061	Δ(*ara-leu*)*7697* Δ(*lac*)*X74*	Laboratory collection
KSK782	MC1061 λKSPL1 (*tcpP-lacZ* wild-type classical)	21
KSK864	MC1061 λKSPL2 (*tcpP-lacZ* wild-type El Tor)	This work
GK334	MC1061 λGK91 (*tcpP-lacZ* C4/C3 control)	This work
GK335	MC1061 λGK92 (*tcpP-lacZ* C4/E3 chimera)	This work
GK336	MC1061 λGK93 (*tcpP-lacZ* E4/C3 chimera)	This work
GK337	MC1061 λGK94 (*tcpP-lacZ* E4/E3 control)	This work
GK370	MC1061 λGK101 (*tcpP-lacZ* classical A→G change at position −65)	This work
GK372	MC1061 λGK102 (*tcpP-lacZ* classical G→T change at position −7)	This work
GK407	MC1061 λGK109 (*tcpP-lacZ* El Tor G→A −66)	This work
GK409	MC1061 λGK111 (*tcpP-lacZ* classical C→A change at position −13)	This work
GK411	MC1061 λGK112 (*tcpP-lacZ* classical A addition at position −19)	This work
GK441	MC1061 λGK117 (*tcpP-lacZ* class A→C change at position −65)	This work
GK442	MC1061 λGK118 (*tcpP-lacZ* El Tor G→C change at position −66)	This work
GK443	MC1061 λGK119 (*tcpP-lacZ* classical A→T change at position −65)	This work
GK444	MC1061 λGK120 (*tcpP-lacZ* El Tor G→T change at position −66)	This work
Expression plasmids		
pKAS107	pMMB66EH *aphA* (classical) Ap^r	33
pKAS117	pMMB66EH *aphB* (classical) Ap^r	21
pKAS121	pBAD-TOPO *aphB* (classical) Kan^r	This work
pKAS126	pBAD-TOPO *aphA* (classical) Kan^r	This work

MATERIALS AND METHODS

Bacterial strains and growth. The *V. cholerae* and *Escherichia coli* strains and plasmids analyzed in this study are listed in Table 1. Strains were maintained at −70°C in LB medium (23) containing 30% (vol/vol) glycerol. Antibiotics were used at the following concentrations in LB medium: ampicillin, 100 μg/ml; kanamycin, 45 μg/ml; polymyxin B, 50 IU/ml; and streptomycin, 1 mg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used in LB agar at 40 μg/ml.

Identification of the *tcpPH* transcriptional start site. El Tor strain GK184 was generated by introducing the Δ*aphA1* deletion from pGKK35 into GK138 (KSK725 Δ*aphB1*) (21). Total RNA was isolated from GK184 and KSK805 carrying both pKAS107 and pKAS121 after growth for 4 h in LB medium (pH 6.5) at 30°C in the presence of 0.2% arabinose and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). RNA was purified on an RNeasy column (Qiagen) and subjected to 5′ rapid amplification of cDNA ends (RACE) (Gibco BRL). Briefly, first-strand cDNA synthesis was carried out with 1 μg of RNA, the *tcpP*-specific primer TP-Bam (33), and reverse transcriptase. The cDNA was purified on a PCR purification column (Qiagen), and a poly(dC) tail was added to the 3′ end by use of terminal deoxynucleotidyl transferase. PCR of the cDNA was carried out initially using a 5′ RACE-abridged anchor primer (5′-GGCCACGCGTCG ACTAGTACGGGIIGGGIIGGGIIG) and the *tcpP* nested primer TP-Kpn (5′-GATCGGGTACCCCGGCTAATTCATGTTGATACC). PCR was carried out subsequently with a 5′ RACE-abridged universal amplification primer (5′-GGC CACGCGTCGACTAGTAC) and a second *tcpP* nested primer, TP-Kpn2 (5′-G ATCGGGTACCTCCACCAAATCACAGGTAGC). The resulting DNA products were sequenced using the ABI PRISM Dye System (Perkin-Elmer).

Introduction of a termination codon into the TcpP coding region. The termination codon (TAA) was introduced into the TcpP coding region 24 bp downstream of the first potential ATG by converting a C to a T and simultaneously creating a restriction site for *Ase*I (see Fig. 2). Two overlapping PCR products containing the desired change (underlined) were amplified from classical biotype DNA using primer pair I-Eco2 (5′-GATCGGAATTCTCTAGAGTACCAATAT CTGTAAAC) and P-Ase2 (5′-GATCGCTCTTCGAATAAATCACGCGGACA TACC) and primer pair P-Ase1 (5′-GATCGCTCTTCGATTAATTTCCCGAT AACCTTTGG) and TP-Kpn. Sites for the restriction enzyme *Ear*I, which cuts outside of its recognition sequence, were incorporated into the P-Ase primers so as to create a seamless junction with no unnatural base pairs (29) upon ligation of the two PCR products into pKAS46 (31). The resulting plasmid, pKAS148, was sequenced, and the mutation was introduced into *V. cholerae* by allelic exchange.

Construction of *V. cholerae tcpPH* promoter replacements. The classical and El Tor *tcpPH* promoter deletion plasmids, pGKK79 and pGKK63, respectively, each contain two DNA fragments of approximately 500 bp which flank the *tcpPH* promoter region (see Fig. 3A) in a derivative of pKAS46 (31), pKAS125, lacking the unique *Sca*I site in the *bla* gene. The upstream fragment, amplified with primer pair I-Eco (5′-GATCGGAATTCATAGTGAGAACGTGTTGCCC) and I-ScaNot (5′-GATCGGCGGCCGCTTATCACGAAGTACTCCGTG), extends from the *Sca*I site 34 bp from the initiation codon of the divergently

transcribed *tcpI* gene into the *tcpI* coding region. The downstream fragment, amplified with primer pair TP-Not1 (5′-GATCGGCGGCCGCTTTCCCGATA ACCTTTGGTGG) and TP-Kpn, lies within the *tcpP* coding sequence (from +40 relative to the start site of *tcpPH* transcription). Introduction of these plasmids into the classical and El Tor *tcpP-lacZ* fusion strains KSK618 and KSK725, respectively, generated the Δ*tcpPH* promoter strains GK300 and GK250, respectively. The *tcpPH* promoter replacement plasmids were constructed by reamplifying the downstream *tcpP* fragments using a primer containing an *Ear*I site, TP-EarI (5′-GATCGCTCTTCGATCAATTTCCCGATAACCTTTG), and TP-Kpn. Each of these fragments was ligated with the appropriate promoter region amplified from either classical or El Tor strains using primer pair TP-Ear2 (5′-GATCGCTCTTCGGATAAATCACGCGGACATACC) and I-Sca (5′-TTA TCACGGAGTACTTCGTG), into the plasmids carrying the upstream *tcpI* fragments. Allelic exchange was carried out with the resulting plasmids, pGKK83, pGKK84, pGKK85, and pGKK86, in the classical and El Tor Δ*tcpPH* promoter strains GK300 and GK250. The *tcpPH* promoter regions of the resulting strains were sequenced.

Construction of *E. coli tcpP-lacZ* fusion strains. The *E. coli* strain carrying the El Tor *tcpP* promoter-*lacZ* fusion, KSK864, was constructed in a manner similar to that described previously for the classical *tcpP* promoter-*lacZ* fusion strain KSK782 (21). Briefly, a 1.3-kb fragment encompassing a region from *tcpI* to *tcpP* was amplified from El Tor DNA using primer pair I-Xba (33) and TP-BamE (21). This fragment was ligated into the *lacZ* operon fusion vector pRS415 to generate pGKK41. The resulting fusion was recombined onto λRS45, generating λKSPL2, and then integrated into the chromosome of *E. coli* MC1061. To construct the classical and El Tor *tcpP* promoter-*lacZ* chimera constructs, the 1.3-kb fragments present in the wild-type fusions were PCR amplified from either biotype as two 650-bp fragments joined at positions −74 and −75, respectively, relative to the start of *tcpPH* transcription using the restriction enzyme *Ear*I (see Fig 4A). The classical C4 and C3 fragments were amplified with primer pair EarC4 (5′-GATCGCTCTTCGTGATAATGAGAACTCGATTG) and I-Eco2 and with primer pair EarC3 (5′-GATCGCTCTTCGTCAACTGCAAA ATTAGATTG) and TP-Bam, respectively. The El Tor E4 and E3 fragments were amplified with primer pair EarE4 (5′-GATCGCTCTTCGTGATAATGA GAACTTAATTGC) and I-Eco2 and with primer pair EarE3 (5′-GATCGCTC TTCGTCAACTGCAGAATTAGATTG) and TP-BamE, respectively. The resulting fragments were ligated into pRS415, generating pGKK91, pGKK92, pGKK93, and pGKK94, and introduced into *E. coli* MC1061. The *tcpPH* promoter regions of the resulting strains were sequenced.

Introduction of single base-pair changes into *E. coli tcpP-lacZ* fusions. Single base pairs within the classical or El Tor *tcpPH* promoters were converted to those of the other biotype by incorporating the changes into primers (underlined in sequences shown below) containing a restriction site for *Ear*I and performing a seamless cloning strategy similar to that described above (see Fig. 5A). Conversion of classical A at position −65 to G was carried out by amplification with primer pair EarC5 (5′-GATCGCTCTTCGTCAACTGCAGAATTAGATTGC) and TP-Bam. The resulting C5 fragment was ligated with the classical C4 fragment from above at position −74 to generate pGKK101. The classical C9 fragment containing the addition of A at position −19 was generated with primer pair EarC9 (5′-GATCGCTCTTCGTCTTAATCATAACGACCCATTTTTTTTT ATG) and I-Eco2. The classical C8 fragment containing the C-to-A change at position −13 was generated with EarC8 (5′-GATCGCTCTTCGTCTTAATCA TAACTACCCATTT) and I-Eco2. The classical C7 fragment containing the G-to-T change at position −7 was generated with EarC7 (5′-GATCGCTCTTC GTCTTAATAATAACGACCCATT) and I-Eco2. Each of these fragments was ligated with a classical C6 fragment generated with primer pair EarC6 (5′-GA TCGCTCTTCGAGAAAATGTAAAGTAATGGGG) and TP-Bam at position −2 to obtain plasmids pGKK112, pGKK111, and pGKK102, respectively.

Conversion of El Tor G at position −66 to A was carried out by amplification with primer pair EarC3 and TP-BamE. The resulting E8 fragment was ligated with the El Tor E4 fragment from above at position −75 to generate pGKK109. To introduce either T or C into positions −65 and −66 of the classical and El Tor promoters, respectively, classical C10 and C11 fragments, respectively, were generated with primer TP-Bam and primer EarC10 (5′-GATCGCTCTTCGTC AACTGCATAATTAGATTGC) or EarC11 (5′-GATCGCTCTTCGTCAACT GCACAATTAGATTGC). These fragments were each ligated with the classical C4 fragment from above at position −74 to generate pGKK119 and pGKK117, respectively. Similarly, El Tor E10 and E11 fragments were amplified with primer TP-BamE and EarC10 or EarC11. These fragments were each ligated with the El Tor E4 fragment from above at position −75 to generate pGKK120 and pGKK118, respectively. The *tcpPH* promoter regions of the resulting strains were sequenced.

Introduction of single base-pair changes into *V. cholerae tcpPH*. The fragment of DNA containing the classical A→G change at position −65 was isolated from pGKK101 and inserted into pKAS32 (31), and that containing the El Tor G→A change at position −66 was isolated from pGKK109 and inserted into pKAS46 (31). The resulting plasmids, pGKK110 and pGKK121, respectively, were used for allelic exchange into classical and El Tor strains. The *tcpPH* promoter regions of the resulting strains were sequenced.

Expression plasmids. A Km^r fragment was inserted into the *aphB* and *aphA* expression plasmids pKAS118 and pKAS119 (21) to generate pKAS121 and pKAS126, respectively (Table 1).

β-Galactosidase assays. β-Galactosidase assays (23) with *V. cholerae tcpP-lacZ* fusions were carried out after growth for 4 h in either LB medium (pH 6.5) at 30°C with rotation or in AKI medium at 37°C without rotation. Overnight cultures of *E. coli tcpP-lacZ* fusions in LB medium (pH 7.0) at 37°C were diluted 1:100 into fresh medium, induced after 2 to 3 h with arabinose and/or IPTG, and assayed after 4 h.

CT assays. GM1 ganglioside enzyme-linked immunosorbent CT assays (12) were carried out after overnight growth in LB medium (pH 6.5) at 30°C.

Immunoblot analysis. Cell extracts prepared from the overnight cultures used for CT assays were subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with anti-TcpA antibody (34), and visualized using the enhanced chemiluminescence detection system (Amersham).

RESULTS

Activation of the El Tor *tcpPH* promoter by AphB is significantly reduced relative to that of the classical promoter. AphA and AphB are both required for transcriptional activation of the *tcpPH* promoter in the classical and El Tor biotypes of *V. cholerae* (21, 33). Strains of the El Tor biotype show reduced expression of the *tcpPH* operon relative to classical biotype strains in vitro (21, 27). The introduction of a plasmid expressing *aphB* into El Tor strains increased the expression from this operon to a level similar to that in classical strains (21). The finding that the expression of *aphB* was similar in both biotypes (21) suggested that some aspect of AphB function might be responsible for the reduced expression of *tcpPH* in El Tor biotype strains. To address whether AphA and AphB function differently at the classical and El Tor *tcpPH* promoters to activate transcription, an El Tor *tcpP-lacZ* Δ*aphA* Δ*aphB* double mutant, GK184, was constructed and compared with a classical *tcpP-lacZ* Δ*aphA* Δ*aphB* double mutant, KSK805, in the presence of plasmids expressing either AphA or AphB (Fig. 1A). Although AphA expressed from plasmid pKAS107 activated both biotype promoters more or less equally, approximately 3- to 5-fold, AphB from pKAS121 activated the classical promoter more than 20-fold, whereas it activated the El Tor promoter only 4-fold. These results indicate that the transcriptional activation of *tcpPH* by AphB is significantly reduced in El Tor biotype strains relative to classical strains.

To rule out possible biotype-specific effects on the activity of AphB or its production from pKAS121, a similar experiment was carried out with *E. coli* with classical (KSK782) and El Tor (KSK864) *tcpP-lacZ* fusions crossed onto λ lysogens (Fig. 1B). AphA from plasmid pKAS126 activated both fusions more or less similarly, between 6- and 12-fold, whereas AphB from pKAS117 activated the classical fusion 40-fold and the El Tor fusion only 2-fold. These results further indicate that the ability of AphB to activate the transcription of the El Tor *tcpPH* promoter is reduced compared to that of the classical promoter.

Determination of the AphB-dependent *tcpPH* transcriptional start site. The start of AphB-dependent *tcpPH* transcription was determined using 5′ RACE (10). In this procedure, RNA was isolated from cultures of *V. cholerae* KSK805 (classical) and GK184 (El Tor) expressing both *aphA* and *aphB* from plasmids. Synthesis of cDNA was carried out using an antisense primer within the *tcpP* gene, and a homopolymeric tail was added to the resulting 3′ end. The cDNA was then amplified by PCR using tail-specific primers and nested *tcpP* primers. A prominent PCR product of approximately 500 bp was observed from both the classical and the El Tor strains; this size corresponded to the size of a product expected for a start site upstream of *tcpP* (data not shown). Direct sequencing of the DNA products revealed an identical start site for both the classical and the El Tor strains: an A residue located 13 bp upstream of the first potential *tcpP* translational initiation codon (Fig. 2). Examination of the 5′ upstream region revealed

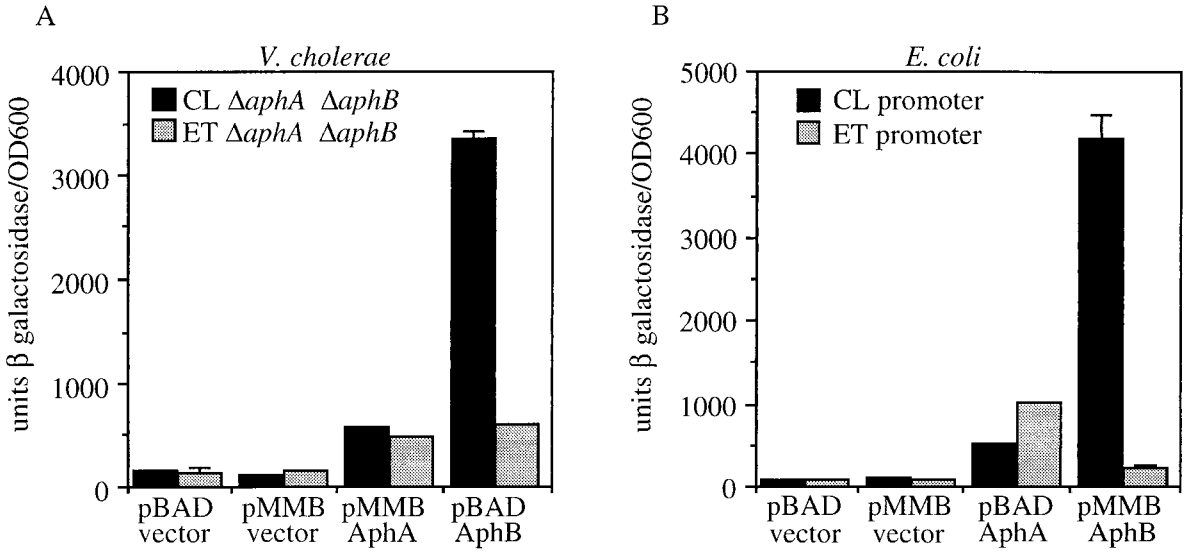

FIG. 1. Differential activation of classical and El Tor biotype *tcpP-lacZ* fusions by AphB. (A) *V. cholerae* cultures were grown in LB medium (pH 6.5) at 30°C. Those with pMMB66EH or pKAS107 (AphA) contained 1 mM IPTG, and those with pBAD or pKAS121 (AphB) contained 0.2% arabinose. Black bars, KSK805 (classical [CL]) Δ*aphA1* Δ*aphB1*; gray bars, GK184 (El Tor [ET]) Δ*aphA1* Δ*aphB1*. (B) *E. coli* cultures were grown in LB medium (pH 7.0) at 37°C. Those with pBAD or pKAS126 (AphA) were induced with 0.2% arabinose, and those with pMMB66EH or pKAS117 (AphB) were induced with 1 mM IPTG. Black bars, KSK782 (classical promoter); gray bars, KSK864 (El Tor promoter). Error bars show standard deviations.

the presence of putative −35 (TAGACA) and −10 (TAT[G or T]AT) sequences (boxed in Fig. 2) separated by 16 bp in the classical promoter and 17 bp in the El Tor promoter. For both regions, five out of six positions matched the *E. coli* consensus sequence.

A potential ATG codon for TcpP translation initiation is located 13 bp downstream from the start site identified above. To rule out the possibility that a second ATG codon, located 85 bp downstream from the first, is actually used for TcpP initiation, a termination codon was introduced 24 bp downstream of the first ATG codon by converting a C to a T. This change converts a CAA codon to TAA and simultaneously creates a restriction site for *Ase*I (Fig. 2). Since the introduction of this termination codon abolished *tcpPH*-mediated activation of virulence gene expression, as indicated by significantly reduced β-galactosidase production from a *V. cholerae* strain carrying a *ctx-lacZ* fusion, strain KSK1019 (data not shown), these results indicate that the second ATG codon is not used for TcpP initiation. It remains possible, however, that a GTG codon located 15 bp downstream of the first ATG codon is used for TcpP initiation. Neither of these potential start codons appears to have a consensus ribosome binding site.

Exchange of the *tcpPH* promoter between classical and El Tor biotypes. The observation that AphB strongly activates the classical but not the El Tor *tcpPH* promoter in *E. coli* suggests that the biotype specificity of *tcpPH* expression in *V. cholerae* is the result of differences in the promoters rather than in the activities of AphB. A comparison of the nucleotide sequence of the region between the upstream divergently transcribed *tcpI* gene and *tcpP* in classical and El Tor biotypes reveals close to 50 base-pair differences. To determine if these differences influence biotype-specific expression of *tcpPH* in *V. cholerae*, the entire region between the *tcpI* and *tcpP* genes (from a *Sca*I site 34 bp downstream of the *tcpI* initiation codon into the *tcpP* gene at position +40) was exchanged between the two biotypes (Fig. 3A). To accomplish this, it was first necessary to delete this entire region from the chromosomes of both *tcpP-lacZ* strains by allelic exchange. The region from the other biotype was then

FIG. 2. Nucleotide sequence of the *tcpPH* promoter. The El Tor residues which differ from the classical residues are shown below the corresponding (underlined) residue. The start of AphB-dependent transcription is indicated by the bold A at +1. Where certain positions differ between the two biotypes relative to the start of transcription, the designations CL for classical and ET for El Tor are used. Boxes delineate putative −35 and −10 promoter consensus sequences. The asterisk denotes the C which was changed to a T to create the stop codon; the thick underlining shows the resulting *Ase*I restriction site.

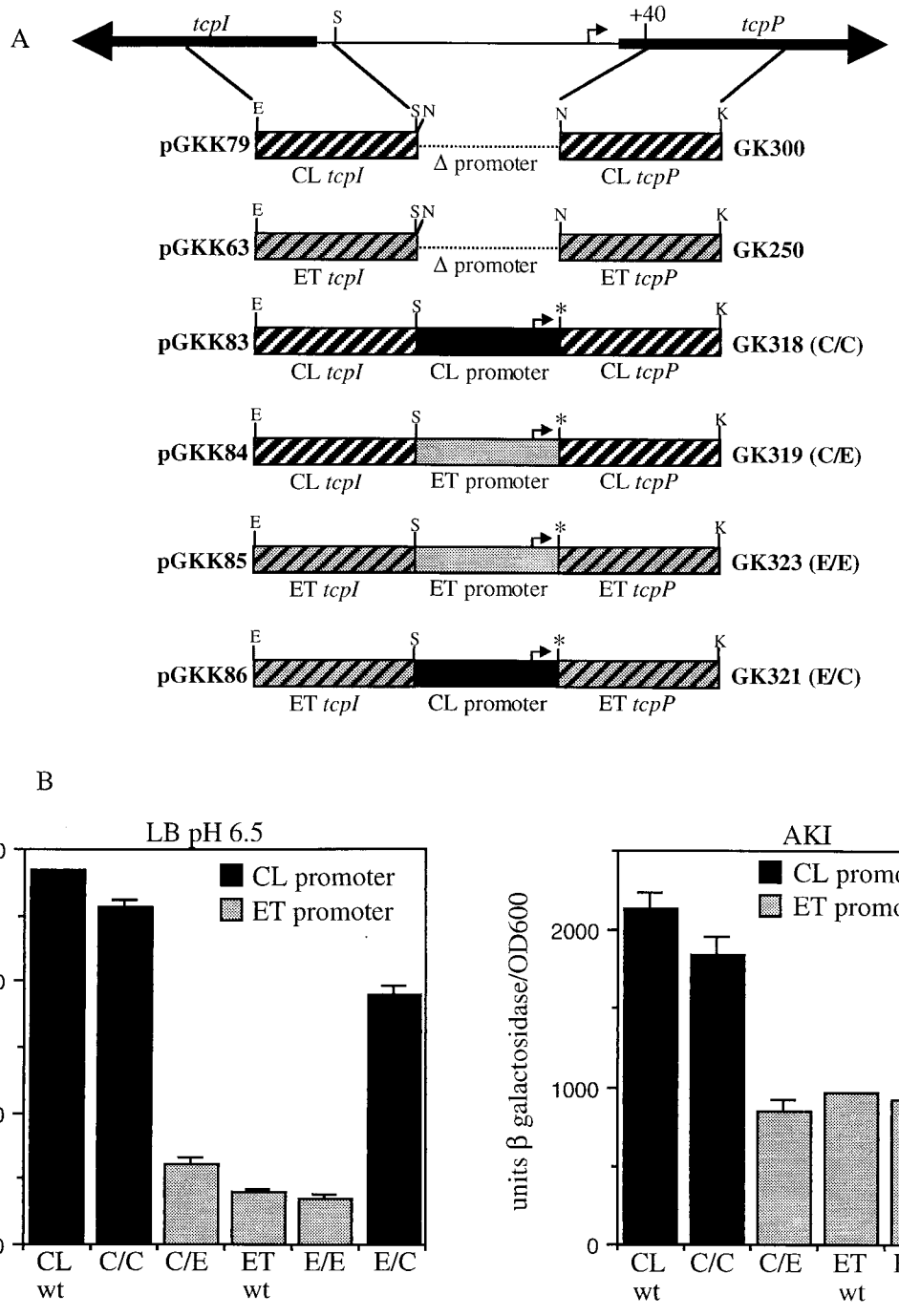

FIG. 3. Reciprocal exchange of the classical and El Tor *tcpPH* promoter regions in *V. cholerae*. (A) Promoter deletion and replacement plasmids and strains. The divergently transcribed coding regions of *tcpI* and *tcpP* are indicated by the large black arrows at the top, and the intergenic region is shown by the solid line. The small arrow denotes the start of the *tcpPH* message. Striped black-white and gray-black boxes represent the regions of *tcpI* and *tcpP* from classical and El Tor, respectively, present in the plasmids (indicated at the left), and dotted lines represent intervening DNA which was deleted. Solid black and gray boxes represent classical and El Tor promoter regions, respectively. The strains generated by allelic exchange from the plasmids are indicated to the right. CL, classical; ET, El Tor; C/C, classical with classical promoter; C/E, classical with El Tor promoter; E/E, El Tor with El Tor promoter; E/C, El Tor with classical promoter. E, *Eco*RI; S, *Sca*I; N, *Not*I; K, *Kpn*I. The asterisk shows the position of the seamless junction created using *Ear*I. (B) Expression of *tcpP-lacZ*. Cultures were grown in LB medium (pH 6.5) at 30°C (left) and in AKI medium at 37°C (right). Black bars, strains with classical (CL) promoter regions: KSK618 (wild type [WT]); GK318 (C/C control); GK321 (E/C hybrid). Gray bars, strains with El Tor (ET) promoter regions: KSK725 (wild type); GK323 (E/E control); GK319 (C/E hybrid). Error bars show standard deviations.

introduced into each deletion strain. The various promoter deletion and replacement plasmids used for the strain constructions are shown in Fig. 3A. Each region that was deleted was also replaced with the region from the same biotype strain to ensure that wild-type expression specific for that biotype was restored.

The results of the *tcpPH* promoter exchange experiment are shown in Fig. 3B. In LB medium (pH 6.5) at 30°C, conditions

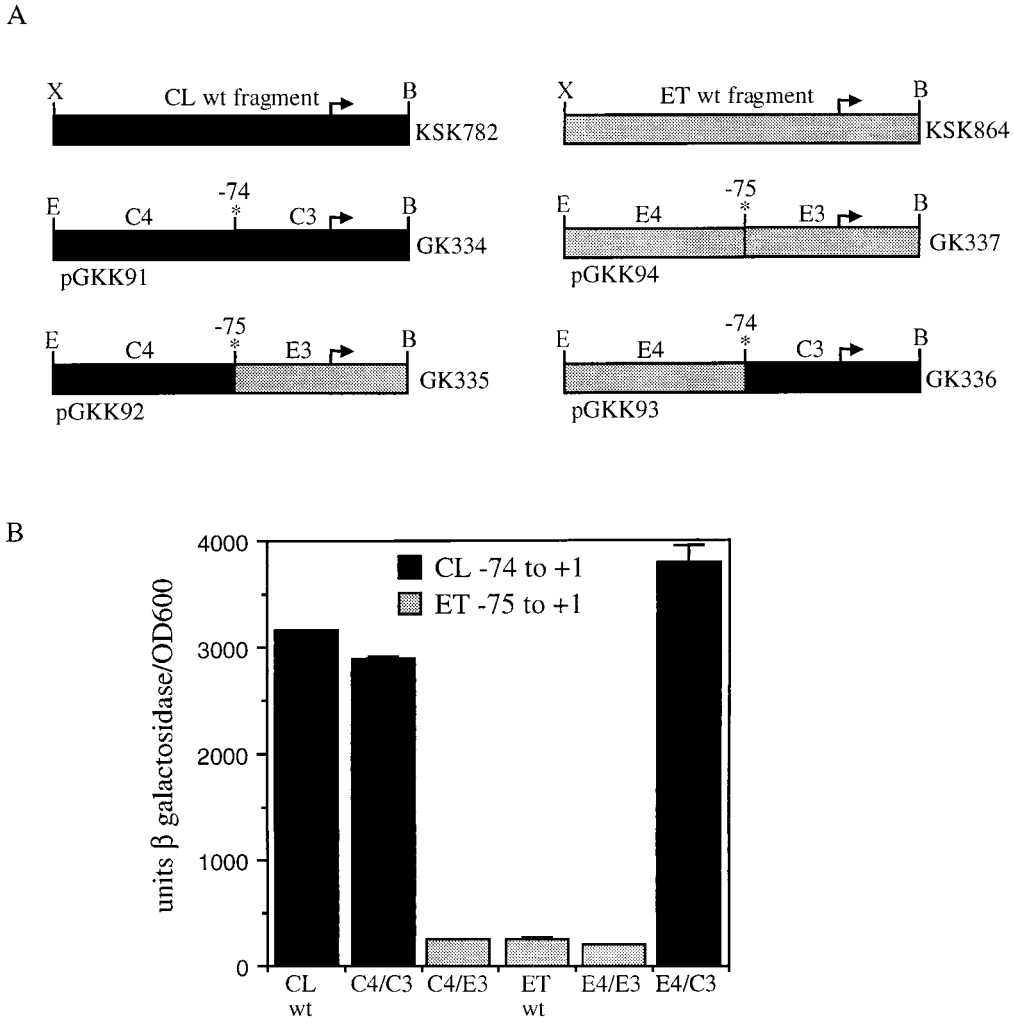

FIG. 4. AphB-dependent expression of classical and El Tor biotype *tcpP-lacZ* promoter chimeras in *E. coli*. (A) PCR-generated *V. cholerae* DNA fragments cloned in pRS415. Black and gray boxes represent classical (CL) and El Tor (ET) DNAs, respectively. Arrows denote the start and direction of transcription. The positions where the two fragments were joined after *Ear*I digestion are shown by the asterisk. *E. coli* lysogens generated from the various constructs are indicated to the right. wt, wild type; X, *Xba*I; B, *Bam*HI; E, *Eco*RI. (B) Expression of *tcpP-lacZ*. Strains containing pKAS117 (AphB) were grown in LB medium (pH 7.0) at 37°C and induced with 1 mM IPTG. Black bars, strains with the classical region from positions −74 to +1: KSK782 (wild type [wt]); GK334 (C4/C3 control); GK336 (E4/C3 chimera). Gray bars, strains with the El Tor region from positions −75 to +1: KSK864 (wild type); GK337 (E4/E3 control); GK335 (C4/E3 chimera). Error bars show standard deviations.

optimal for *tcpPH* expression in the classical biotype, the wild-type classical fusion, KSK618, produced approximately 3,000 U of β-galactosidase, and the wild-type El Tor fusion, KSK725, produced about 400. Each of the control replacement strains, GK318 (classical with classical promoter) and GK323 (El Tor with El Tor promoter) for El Tor, showed levels of expression comparable to those of the corresponding wild-type fusion strains. Insertion of the El Tor *tcpPH* promoter into the classical biotype, strain GK319 (classical with El Tor promoter), decreased the expression of the fusion to a level close to that observed with the wild-type El Tor strain. Conversely, insertion of the classical *tcpPH* promoter into the El Tor biotype, strain GK321 (El Tor with classical promoter), increased expression to close to the wild-type classical strain level. A similar promoter-specific result was observed after growth of the strains under AKI medium conditions, which are known to induce *tcpPH* expression in El Tor strains (Fig. 3B). Under these conditions, however, the strains containing the El Tor promoters still displayed reduced *tcpPH* expression relative to those with the classical promoters. These results indicate that sequence differences in the respective promoter regions are important for the biotype specificity of *tcpPH* expression in *V. cholerae*.

Analysis of biotype *tcpP-lacZ* promoter chimeras in *E. coli*. To discern which regions within the classical and El Tor *tcpPH* promoters are responsible for biotype-specific activation by AphB, classical and El Tor *tcpP-lacZ* promoter chimeras were constructed and analyzed in *E. coli*. To accomplish this, the 1.3-kb fragments present in the wild-type *E. coli tcpP-lacZ* fusions were PCR amplified from the classical and El Tor biotypes as two approximately 650-bp fragments using overlapping primers at positions −74 and −75, respectively, relative to the start site of *tcpPH* transcription. As shown in Fig. 2, these positions separated a promoter-proximal region containing four nucleotide differences from a distal region containing all of the remaining nucleotide differences. The two fragments from the different biotypes were then seamlessly joined together in pRS415 (Fig. 4A). Fragments from the same biotype

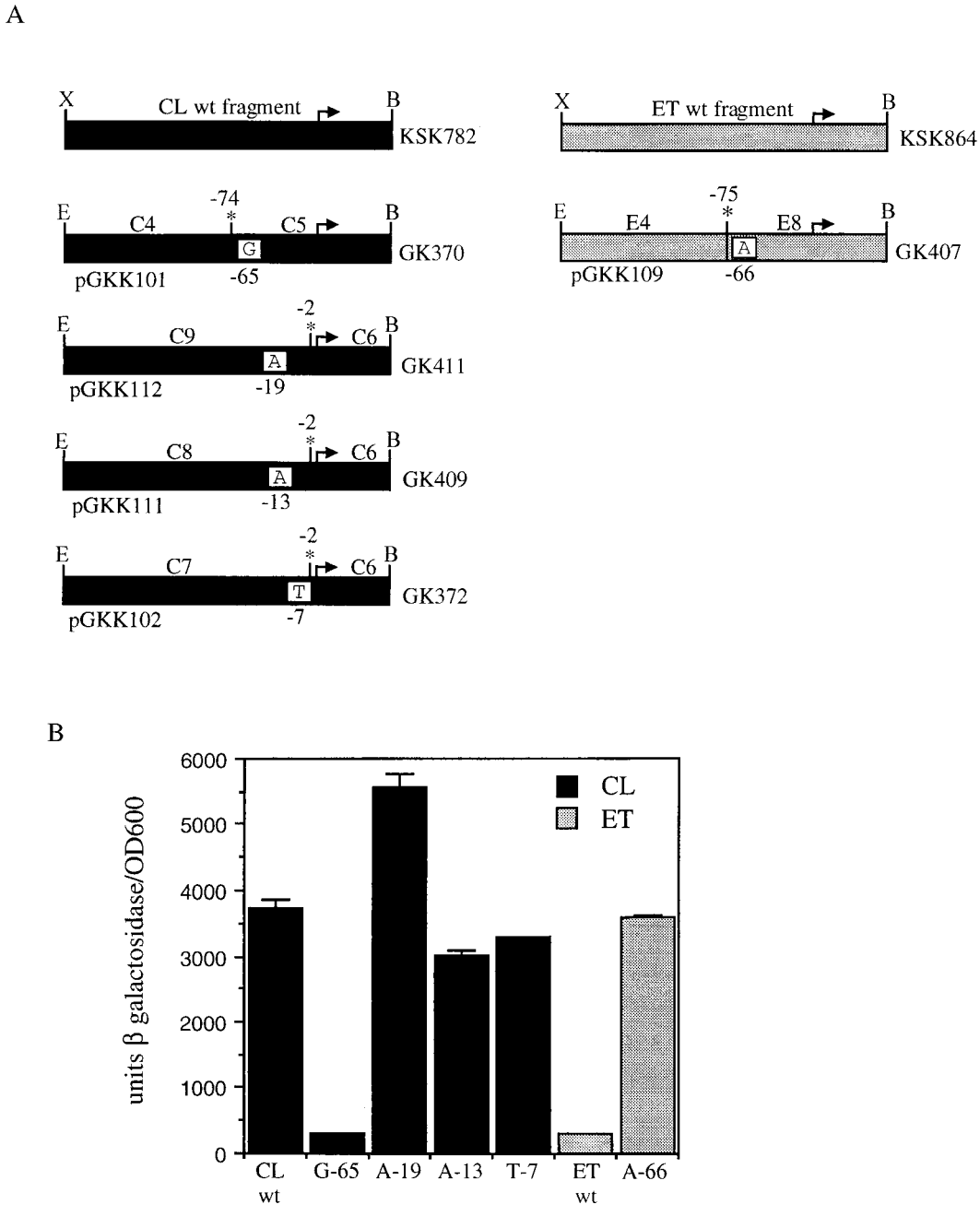

FIG. 5. AphB-dependent expression in *E. coli tcpP-lacZ* fusions containing single base-pair alterations. (A) See the legend to Fig. 4A. Single base-pair alterations are indicated in white boxes with the positions from the start of transcription shown below. (B) Expression of *tcpP-lacZ*. Strains containing pKAS117 (AphB) were grown as described in the legend to Fig. 4B. Black bars, strains with classical (CL) DNA: KSK782 (wild type [wt]); GK370 (A→G change at position −65); GK411 (addition of A at position −19); GK409 (C→A change at position −13); GK372 (G→T change at position −7). Gray bars, strains with El Tor (ET) DNA: KSK864 (wild type); GK407, (G→A change at position −66). Error bars show standard deviations.

were also joined to ensure that wild-type expression was restored. The resulting fusions were then introduced into the *E. coli* chromosome and analyzed in the presence of AphB. Figure 4B shows that the region downstream of position −74 or −75 determines the biotype specificity of *tcpPH* expression. When the upstream classical fragment was joined with the downstream El Tor fragment, resulting in strain GK335 (C4 fragment + E3 fragment), AphB-dependent expression was decreased to a level virtually identical to that seen with the wild-type El Tor fusion. Conversely, when the upstream El Tor fragment was joined with the downstream classical fragment, resulting in strain GK336 (E4 fragment + C3 fragment), expression was increased to a level similar to that seen with the wild-type classical fusion. These results suggest that either one or several of the four base-pair differences between classical and El Tor strains in the region from positions −74 or −75 to +1 of the *tcpPH* promoter (Fig. 2) are responsible for biotype-specific activation by AphB.

Introduction of single-base-pair changes into *E. coli tcpP-lacZ* fusions. To elucidate which of the four base-pair differ-

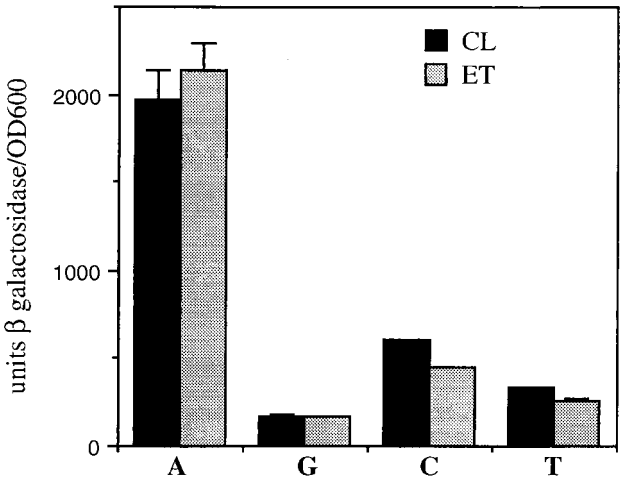

FIG. 6. AphB-dependent expression in *E. coli tcpP-lacZ* fusions containing A, G, C, or T at position −65 or −66. Strains containing pKAS117 (AphB) were grown as described in the legend to Fig. 4B. Black bars, classical (CL) fusions: KSK782 (wild type, A at position −65); GK370 (G at −65); GK441 (C at −65); GK443 (T at −65). Gray bars, El Tor (ET) fusions: GK407 (A at position −66); KSK864, (wild type, G at −66); GK442 (C at −66); GK444 (T at −66). Error bars show standard deviations.

ences in the region from positions −74 or −75 to +1 of the *tcpPH* promoter might be important for biotype-specific expression, the four El Tor residues, G at −66, A at −19, A at −13, and T at −7, were separately introduced in place of the corresponding classical residues (or lack thereof) in an *E. coli tcpP-lacZ* fusion, and the resulting strains were analyzed in the presence of AphB. The constructs containing these single base-pair changes (Fig. 5A) were made by a procedure similar to that used to create the chimeras shown in Fig. 4. Replacement of the classical A at position −65 with G, strain GK370, had a significant effect on AphB-dependent expression, reducing activation from 40- to 4-fold (Fig. 5B). This reduced level of expression was essentially identical to that observed with the wild-type El Tor fusion strain KSK864. The addition of an A at position −19, strain GK411, actually increased *tcpPH* expression somewhat. This result might be expected since the alteration increased the spacing between the −35 and −10 regions from 16 bp to the more optimal distance of 17 bp. Replacement of the classical C at position −13 with A and the G at position −7 with T had no significant effect on AphB-dependent transcriptional activation; both strains, GK372 and GK409, respectively, still showed approximately 40-fold activation by AphB. Thus, of the four El Tor base-pair replacements made in the classical fusion, only the G at position −65 significantly reduced AphB-dependent transcription. When the G at position −66 of the El Tor *tcpP-lacZ* fusion was converted to A, AphB-dependent transcription in the resulting strain, GK426, was activated 40-fold, a level similar to that observed with the classical fusion strain KSK782 (Fig. 5B). These results indicate that the presence of either an A or a G at position −65 or −66 of the classical or El Tor *tcpPH* promoter, respectively, determines biotype-specific expression by influencing the ability of AphB to activate transcription.

Since the presence of an A or a G at position −65 or −66 of the *tcpPH* promoter dramatically influenced the ability of AphB to activate transcription, it was of interest to determine the effect of replacing the base pair at this position with either C or T. Whereas the presence of A or G at position −65 or −66 permitted 40- or 4-fold activation of *tcpPH* transcription by AphB, respectively, replacement of this base pair with either C or T resulted in approximately 10- or 5-fold activation, respectively (Fig. 6). As was observed with A or G, the presence of either C or T produced nearly equivalent effects on AphB-dependent transcription from both biotype promoters. These results indicate that an adenine residue at position −65 or −66 of the *tcpPH* promoter is critical for optimal AphB-dependent activation of transcription.

Influence of A or G at position −65 or −66 on virulence gene expression in *V. cholerae*. To determine the effect in *V. cholerae* of reciprocal exchange between biotypes of the A or G residue at position −65 or −66 of the *tcpPH* promoter on *tcpPH* expression, fragments of DNA containing the single base-pair changes from above were cloned into allelic exchange vectors and introduced into the chromosomes of the Δ*tcpPH* promoter *tcpP-lacZ* fusion strains GK300 and GK250. Classical strain GK404, which has a classical *tcpPH* promoter except for a G at position −65, showed a significantly reduced level of β-galactosidase production similar to that of the wild-type El Tor fusion strain KSK725 in either LB medium (pH 6.5) at 30°C or AKI medium (Fig. 7A). In contrast, β-galactosidase production in El Tor strain GK436, which has an El Tor *tcpPH* promoter except for an A at position −66, mirrored that observed with the wild-type classical fusion strain KSK618. These results support those obtained with *E. coli* indicating that the biotype-specific pattern of *tcpPH* expression is determined by the presence of an A or a G at position −65 or −66 of the *tcpPH* promoter.

To determine the influence of these base-pair changes on the production of TCP and CT in the two biotypes, the G at position −65 in the classical *tcpPH* promoter was introduced into classical strain O395, and the A at position −66 in the El Tor *tcpPH* promoter was introduced into El Tor strain C6706 str2. As shown in Fig. 7B, the presence of a G at position −65 in the classical *tcpPH* promoter, strain KSK1093, reduced the production of TCP in LB medium (pH 6.5) at 30°C to a level undetectable by Western blotting and decreased toxin production from 1,500 ng/ml per unit of optical density at 600 nm (OD_{600}) to less than 10 ng/ml. Conversely, an A at position −66 in the El Tor *tcpPH* promoter, strain KSK1117, increased TCP production to a detectable level in LB medium (pH 6.5) at 30°C and increased toxin production from less than 10 ng/ml/OD_{600} unit to almost 1,400 ng/ml. These results indicate that it is the ability of AphB to activate *tcpPH* expression which largely determines the biotype-specific pattern of virulence gene expression that is observed in *V. cholerae*.

DISCUSSION

The expression of the genes encoding TCP and CT is regulated by a transcriptional cascade involving multiple activator proteins. The AraC regulator ToxT directly activates the transcription of the *tcpA* and *ctx* operons (4, 9, 15). The expression of *toxT* is in turn regulated by two pairs of transmembrane transcriptional activator proteins, ToxRS and TcpPH (7, 13, 24, 26). The expression of the *tcpPH* operon is activated by the LysR regulator AphB in cooperation with a second protein, AphA (21, 33). Each of these activator proteins appears to play a similar role in both disease-causing biotypes of *V. cholerae*. However, the expression of *tcpA*, *ctx*, *toxT*, and *tcpPH* is significantly lower in the El Tor biotype than in the classical biotype under most in vitro culture conditions (8, 27). The overproduction of AphB from a plasmid in the El Tor biotype has recently been observed to dramatically increase *tcpPH* and *tcpA* expression to close to classical levels in vitro (21). This result, together with the finding that the expression of *aphB* is

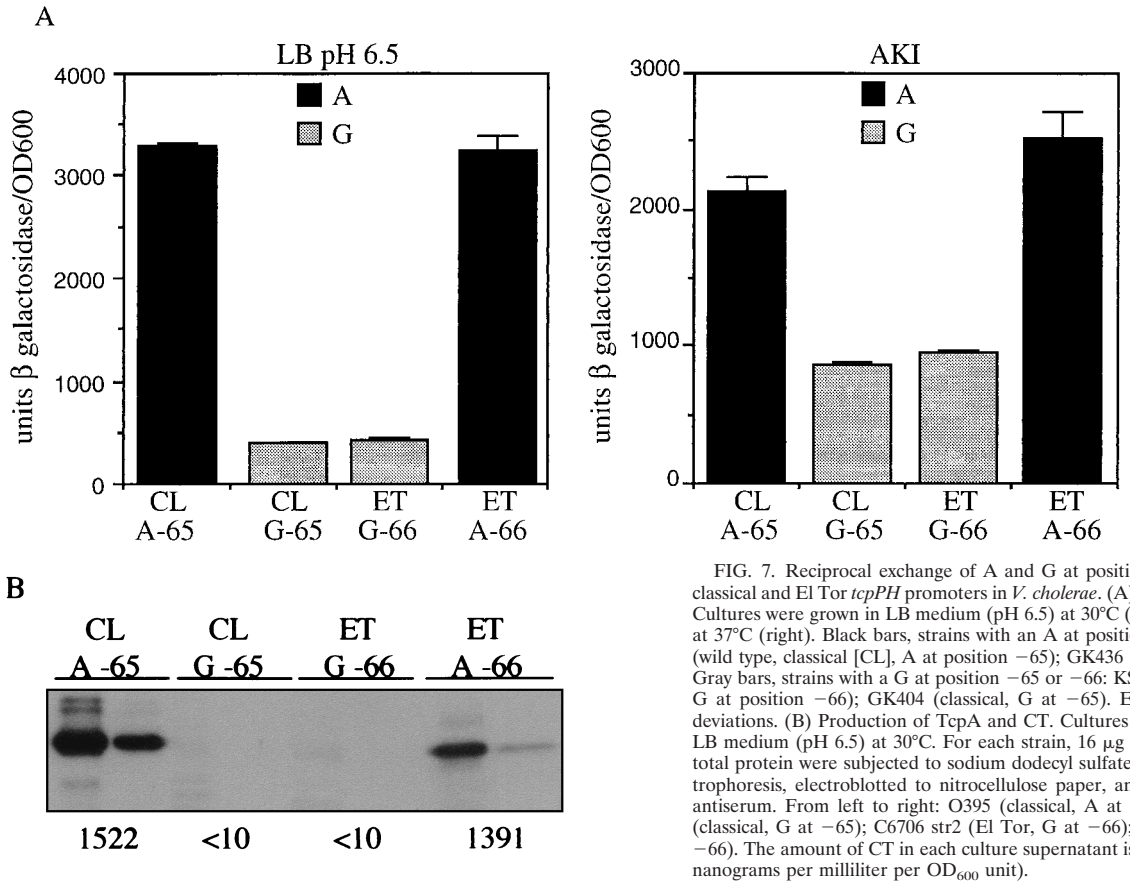

FIG. 7. Reciprocal exchange of A and G at positions −65 and −66 of the classical and El Tor *tcpPH* promoters in *V. cholerae*. (A) Expression of *tcpP-lacZ*. Cultures were grown in LB medium (pH 6.5) at 30°C (left) and in AKI medium at 37°C (right). Black bars, strains with an A at position −65 or −66: KSK618 (wild type, classical [CL], A at position −65); GK436 (El Tor [ET], A at −66). Gray bars, strains with a G at position −65 or −66: KSK725 (wild type, El Tor, G at position −66); GK404 (classical, G at −65). Error bars show standard deviations. (B) Production of TcpA and CT. Cultures were grown overnight in LB medium (pH 6.5) at 30°C. For each strain, 16 μg (left) and 3 μg (right) of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted to nitrocellulose paper, and probed with anti-TCP antiserum. From left to right: O395 (classical, A at position −65); KSK1093 (classical, G at −65); C6706 str2 (El Tor, G at −66); KSK1117 (El Tor, A at −66). The amount of CT in each culture supernatant is shown at the bottom (in nanograms per milliliter per OD_{600} unit).

similar in both biotypes (21), raised the possibility that the biotype-specific expression of *tcpPH* might be related to the ability of AphB to activate transcription at each of these promoters.

To address this issue, experiments carried out with both *V. cholerae* and *E. coli* showed that when expressed from a plasmid, AphB alone activated the classical *tcpPH* promoter 20- to 40-fold, whereas it activated the El Tor promoter only 2- to 4-fold. That AphB showed reduced transcriptional activation of the El Tor *tcpPH* promoter relative to the classical promoter in *E. coli* suggested that the molecular basis for this difference might be at the level of the promoter rather than a biotype-specific influence over the activity of the protein. Indeed, replacement of the entire region between the *tcpI* and *tcpP* genes in *V. cholerae* with that of the other biotype showed that it is the *tcpPH* promoter itself which determines the biotype-specific pattern of expression. Biotype promoter chimera constructs in *E. coli* localized the region of the *tcpPH* promoter responsible for this difference to within 75 bp of the start of transcription. Of the four base-pair differences between the two biotypes in this region from positions −75 to +1, only conversion of the A at position −65 in the classical promoter to the El Tor G dramatically reduced AphB-dependent expression of *tcpPH* to the level typically observed with El Tor. The presence of a C or a T residue in either biotype promoter at this position only marginally increased AphB-dependent expression over that observed with a G residue. These results indicate that an A at position −65 or −66 in the *tcpPH* promoter is optimal for transcriptional activation by AphB.

DNA footprinting studies have shown that many LysR transcriptional regulators bind to promoter regions via a 15-bp dyad sequence with a common structure and location near the −65 region of the promoter (30). Two well-characterized LysR regulators are MetR from *Salmonella enterica* serovar Typhimurium (2, 22, 36) and TrpI from *Pseudomonas* (5, 11). MetR positively regulates several genes which encode enzymes in the methionine biosynthetic pathway. The protein binds to a region upstream of the *metE* transcriptional start site that contains the interrupted dyad TGAA-N5-TTCA from −67 to −55 (36). A similar sequence is also found in the MetR binding regions of the *metH* and *metA* promoters (2, 22). TrpI positively regulates the *trpBA* genes encoding tryptophan synthetase. The protein similarly binds to a region containing an interrupted dyad between −71 and −57 relative to the *trpBA* transcriptional start site: GTgAG-N5-CTgAC (5, 11). Examination of the DNA sequence around position −65 in the classical *tcpPH* promoter revealed the presence of an interrupted dyad from −69 to −53 (TGCAA-N7-TTGCA) that is a potential site for the interaction of AphB with DNA, based on similarities with other LysR-regulated promoters; the A at −65 is in the left arm of the dyad (indicated by the underline). It is possible that the conversion to G at this position reduces the binding of AphB to DNA. Substitution mutations in the conserved dyad arms of the consensus sequence upstream of the *metH* gene have been found to significantly reduce MetR binding and transcriptional activation (2). Experiments are currently in progress to determine whether this position is the actual binding site for AphB in the *tcpPH* promoter.

In *V. cholerae* as well as in *E. coli*, the biotype-specific pattern of *tcpPH* expression is determined by the presence of an A

or a G at position −65 or −66 in the respective promoters. Substitution of the A at position −65 of the classical *tcpPH* promoter with a G decreased the expression of *tcpPH* in LB medium (pH 6.5) at 30°C to a level typically observed with El Tor. In addition, the production of TCP and CT was reduced under these conditions to levels comparable to those observed with El Tor. This classical A→G (position −65) mutant also failed to autoagglutinate. These results suggest that the lower levels of TcpP and TcpH in this mutant prevent the normally high-level expression of *toxT* that occurs with classical strains under these conditions.

Conversely, substitution of the G at position −66 of the El Tor *tcpPH* promoter with an A in *V. cholerae* increased the expression of *tcpPH* to classical levels in LB medium (pH 6.5) at 30°C. The observation that the production of both TCP and CT was increased by the G-to-A substitution suggests that the expression of *toxT* has been increased as a consequence of the higher levels of TcpP and TcpH present in the cells. However, the level of TcpA in the El Tor G→A (position −66) mutant, although dramatically increased from the level in the wild-type strain, still appeared to be approximately fivefold lower than that observed in the classical strain. In addition, the mutant did not strongly autoagglutinate in LB medium (pH 6.5) at 30°C like the classical strain did. This result suggests that additional differences with regard to the expression of *tcpA* may still exist between the two biotype strains. Consistent with this hypothesis, β-galactosidase production in an El Tor *tcpA-lacZ* fusion strain containing a G- to-A substitution at position −66 of the *tcpPH* promoter is approximately twofold lower than that in a wild-type classical *tcpA-lacZ* fusion strain (data not shown). In contrast, a wild-type El Tor *tcpA-lacZ* fusion strain (with a G at position −66 of the *tcpPH* promoter) shows 150-fold lower β-galactosidase production than the classical *tcpA-lacZ* fusion strain. Thus, although the G→A (position −66) substitution in the *tcpPH* promoter dramatically increased the expression of *tcpA-lacZ* in the El Tor strain, this increased level of expression was still lower than that observed with the classical strain. There are a number of nucleotide differences between the classical and El Tor *tcpA* promoters which could potentially influence transcription.

The evolution of toxigenic *V. cholerae* from environmental strains incapable of causing disease involves the acquisition of the TCP-ACF element (18, 19, 20) and the CTXϕ phage (37). It is interesting that ToxR, ToxS, AphA, and AphB are not encoded within either of these elements, yet they play essential roles in activating the expression of virulence genes that are present on them. How the virulence gene promoters on these elements evolved to come under the control of these activator proteins can only be speculated. In addition, it is not obvious why the expression of *tcpPH* in the classical and El Tor biotype strains evolved to become differentially activated by AphB. One possibility is that the reduced virulence of the strain containing a G at position −66 of the *tcpPH* promoter enabled the organism to persist within the host to a greater degree and thus be more efficiently disseminated into the environment.

The work presented here establishes a basis for the differential regulation of virulence gene expression between the two disease-causing biotypes of *V. cholerae*, classical and El Tor. The insights gained from this work will aid in the further analysis of how AphA and AphB interact with the *tcpPH* promoter to influence virulence gene expression.

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